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The prolonged culture of human immunodeficiency virus type 1 in primary lymphocytes increases its sensitivity to neutralization by soluble CD4

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Abstract

Primary strains of human immunodeficiency virus type 1 (HIV-1) are known to adapt to replication in cell lines in vitro by becoming sensitive to soluble CD4 (sCD4) and neutralizing antibodies (NAb). T-cell lines favor isolation of variants that use CXCR4 as a co-receptor, while primary isolates predominantly use CCR5. We have now studied how a primary R5 isolate, CC1/85, adapts to prolonged replication in primary human peripheral blood mononuclear cells (PBMC). After 19 passages, a variant virus, CCcon.19, had increased sensitivity to both sCD4 and NAb b12 that binds to a CD4-binding site (CD4BS)-associated epitope, but decreased sensitivity to anti-CD4 antibodies. CCcon.19 retains the R5 phenotype, its resistance to other NAbs was unaltered, its sensitivity to various entry inhibitors was unchanged, and its ability to replicate in macrophages was modestly increased. We define CCcon.19 as a primary T-cell adapted (PTCA) variant. Genetic sequence analysis combined with mutagenesis studies on clonal, chimeric viruses derived from CC1/85 and the PTCA variant showed that the most important changes were in the V1/V2 loop structure, one of them involving the loss of an N-linked glycosylation site. Monomeric gp120 proteins expressed from CC1/85 and the PTCA variant did not differ in their affinities for sCD4, suggesting that the structural consequences of the sequence changes were manifested at the level of the native, trimeric Env complex. Overall, the adaptation process probably involves selection for variants with higher CD4 affinity and hence greater fusion efficiency, but this also involves the loss of some resistance to neutralization by agents directed at or near to the CD4BS. The loss of neutralization resistance is of no relevance under in vitro conditions, but NAbs would presumably be a counter-selection pressure against such adaptive changes in vivo, at least when the humoral immune response is intact.

Keywords: CD4; Human immunodeficiency virus type 1; CCcon.19

Introduction

Human immunodeficiency virus type 1 (HIV-1) has evolved multiple mechanisms to resist neutralization by antibodies (Burton, 2002; Johnson and Desrosiers, 2002; Moore and Ho, 1995; Moore et al., 1995; Parren et al., 1999; Poignard et al., 2001). These devices are necessary for the virus to persist long enough in vivo to be transmitted to a new host because sexual transmission is such an inefficient process, usually requiring many contacts over a prolonged period (Shattock and Moore, 2003). The humoral immune response to HIV-1 is vigorous; antibodies to the viral envelope glycoproteins (Env) gp120 and gp41 are generated at very high titers and persist throughout the course of infection (Binley et al., 1997; Cheingsong-Popov et al., 1991; Parren et al., 1999; Richman et al., 2003; Wei et al., 2003). Unfortunately, most anti-Env antibodies are unable to neutralize the virus because they fail to bind to functional spikes on infectious virions or infected cells (Herrera et al., 2003; Parren et al., 1999; Poignard et al., 2003). And whenever neutralization does occur successful-

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ly, HIV-1 almost invariably mutates to generate escape variants (Arendrup et al., 1993; Moore et al., 1995; Parren et al., 1999; Reitz et al., 1988; Richman et al., 2003; Wei et al., 2003). These evasion and escape pathways are major obstacles for the immune system in its fight with HIV-1, and they also present serious problems for the design of vaccines based on humoral immunity and of some drugs that prevent HIV-1 entry (Moore and Doms, 2003; Moore and Stevenson, 2000; Parren et al., 1999). For example, the clinical development of soluble CD4 (sCD4) was derailed by the insensitivity of primary isolates to this engineered molecule (Ashkenazi et al., 1991; Daar et al., 1990; Moore and Sweet, 1993; Moore et al., 1992, 1993; Willey et al., 1994). The sCD4 binding site on gp120 is similar, but not identical, to those for neutralizing antibodies (NAb) directed against epitopes proximal to the CD4-binding site (CD4BS) (Olshevsky et al., 1990; Pantophlet et al., 2003; Thali et al., 1992). Resistance to sCD4 may be merely a coincidental consequence of HIV-1's natural defense mechanisms against NAbs to the CD4BS-associated epitopes (Moore and Ho, 1993, 1995).

Although HIV-1 is well equipped to resist the effect of NAbs in vivo, it becomes neutralization-sensitive when cultured in vitro (Clapham and McKnight, 2002; Moore and Ho, 1995; Moore et al., 1992, 1993, 1995; Turner et al., 1992; Wrin et al., 1995). This can be a rapid process, taking only a few replication cycles. Moreover, when a neutralization-sensitive, in vitro-adapted, or engineered virus infects a monkey, or accidentally, a human, then readaptation to the neutralization-resistant phenotype occurs (Beaumont et al., 2001; Cheng-Mayer et al., 1999; Etemad-Moghadam et al., 2000; Si et al., 2001). Neutralization-sensitive viruses simply do not persist in vivo (Johnson et al., 2002, 2003). Neutralization resistance seems, therefore, to be dispensable, but only if antibodies with actual or potential neutralizing activity are absent from the environment in which the virus is replicating.

Does HIV-1 pay a price for its protection from NAbs? In other words, is the virus's rate of replication slowed by the armor that it is, metaphorically, carrying? This has long been hypothesized (Moore and Ho, 1995), but never satisfactorily answered, because most in vitro adaptation experiments have involved the passage of HIV-1 into a permanent cell line to make a T-cell line-adapted (TCLA) strain. T-cell lines almost invariably differ from primary $CD4^+$ T cells in the identity or quantity of the viral co-receptors that they express (Berger et al., 1999; Clapham and McKnight, 2002). While most primary HIV-1 strains have the R5 phenotype, a minor X4 component is often present (Shankarappa et al., 1999). These viruses may be able to emerge fully only when CCR5 is not available (Kinter et al., 1998). Thus, most T-cell lines express the CXCR4 co-receptor, but not CCR5, which allows the isolation of only X4 viruses. Even if the cell line does express CCR5, it usually does so at a level that differs from what is present on primary $CD4^+$ T cells, and the level of CCR5 expression is an adverse influence on the potency of entry inhibitors (Doms, 2000; Lee et al., 1999; Moore and Doms, 2003; Platt et al., 2000; Reeves et al., 2002). Like resistance to neutralizing antibodies, co-receptor usage is determined by the structure of the viral Env complex (Berger et al., 1999; Clapham and McKnight, 2002; Poignard et al., 2001). An additional, but interrelated variable is the amount of CD4 present on the target cell, which can become limiting for entry when co-receptor expression levels are below a certain threshold (Platt et al., 1998). This factor may be particularly relevant to the entry of X4 variants, as these viruses are more sensitive than R5 strains to the cell surface CD4 concentration; indeed, X4 viruses are unable to infect cells expressing very low levels of CD4 (Kozak et al., 1997; Tokunaga et al., 2001). Moreover, HIV-1 uses different methods to attach to different cell types before CD4 and co-receptor engagement, and these attachment processes are also Env-dependent (Mondor et al., 1998). For example, HIV-1 binds to heparin sulfate proteoglycans on several cell lines via gp120, but this is a minor attachment mechanism for primary $CD4^+$ T cells (Ibrahim et al., 1999; Mondor et al., 1998; Patel et al., 1993; Spenlehauer et al., 2001). Consequently, it is hard to discriminate between multiple, sometimes overlapping influences when studying why a primary virus becomes sensitive to NAbs upon passage in vitro. Is it the absence of NAbs that allows the virus to discard its protective armor? Or are other factors involved in adaptation to growth in cell lines also relevant?

To address these questions, we have cultured an R5 primary HIV-1 isolate, CC1/85, for a prolonged period only in primary, mitogen-stimulated human peripheral blood mononuclear cells (PBMC), to minimize the variables involved in vitro adaptation. We found that the adapted virus, CCcon.19, eventually became more sensitive to sCD4 and the b12 NAb, although it retained its resistance to several other monoclonal antibodies (MAbs) and entry inhibitors. CCcon.19 was also less sensitive than CC1/85 to anti-CD4 MAbs. These phenotypic changes were associated with three amino acid substitutions in the V2 region of gp120, one of which eliminated an N-linked glycan site. We believe these findings are consistent with the concept that changes in Env can increase the rate of receptor binding and fusion, but may also cause an increase in neutralization sensitivity. Sometimes this price is worth paying (in vitro), sometimes not (in the face of an effective humoral immune response, in vivo). In the end, HIV-1 strikes the appropriate balance between attacking its receptors and defending itself against the NAbs that try to prevent receptor binding.

Results

Generation of a PBMC-adapted HIV-1 variant

We have previously described the generation of an escape mutant, CC101.19, under the selection pressure of

AD101, a CCR5-specific small molecule inhibitor of HIV-1 entry (Kuhmann et al., in press; Trkola et al., 2002). The creation of this escape variant necessitated the continuous passage of the R5 primary HIV-1 isolate CC1/85 in PBMC in the presence of increasing AD101 concentrations. As a control, CC1/85 was passaged in PBMC from the same donors, but in the absence of AD101. Thus, by the time a virus emerged that was essentially completely resistant to AD101, the control culture contained a CC1/85 variant, CCcon.19, that had been grown continuously in PBMC for 19 weeks (Fig. 1). We reasoned that its extensive history of in vitro replication might have altered the phenotypic properties of CC1/85, despite its maintenance only in primary cells. We designate the CCcon.19 virus as a "primary T-cell adapted" (PTCA) HIV-1 variant.

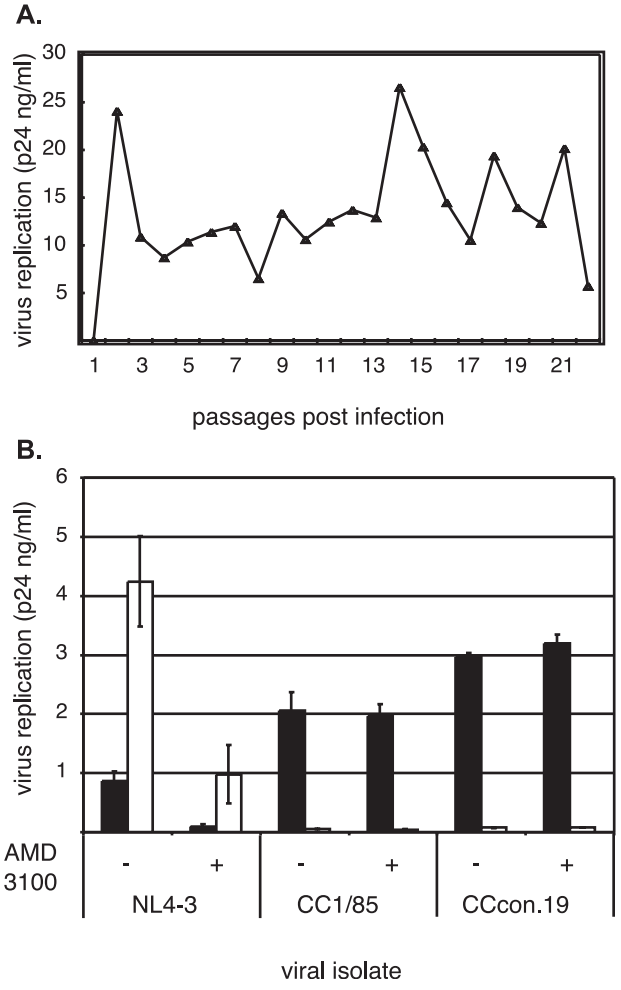

Fig. 1. Generation of CCcon.19, a PBMC-adapted R5 HIV-1 variant. (A) HIV-1 primary isolate CC1/85 was cultured in primary $CD4^+$ T cells. The extent of virus replication (p24 antigen production) at each passage is shown. (B) HIV-1 isolates NL4-3 (X4), CC1/85 (R5), and CCcon.19 were used to infect primary $CD4^+$ T cells from donors expressing wild type CCR5 (filled bars) and donors homozygous for the CCR5-Δ32 mutation (open bars). The amount of p24 antigen produced in the absence and presence of 100 nM AMD3100 is shown. The error bars represent the SEM of values from three independent experiments.

Co-receptor usage profile of PTCA variant CCcon.19

We first determined whether its 19-week passage in PBMC had affected the co-receptor usage profile of CC1/85 (Connor et al., 1997; Trkola et al., 2002). Neither CC1/85 nor CCcon.19 could replicate in PBMC from two different donors who were homozygous for the CCR5-Δ32 allele and so lack CCR5 (Fig. 1B). Hence, both CC1/85 and CCcon.19 absolutely require CCR5 for replication. Furthermore, neither virus could replicate in GHOST-CD4 cells that expressed CXCR4, but both could do so in GHOST-CD4-CCR5 cells (data not shown). The replication of both CC1/85 and CCcon.19 in PBMC were completely inhibited by similar concentrations of the CCR5 inhibitors SCH-C, MAb PA14, and RANTES (Figs. 2A–C, Table 1), but both viruses were insensitive to the CXCR4-specific inhibitor, AMD3100 (Fig. 1B, Table 1). We conclude that the co-receptor usage profile of CC1/85 was unaltered by its passage in PBMC, and that CCcon.19 has retained the original R5 phenotype of CC1/85. We could find no evidence that CCcon.19 can use CXCR4 for entry.

PTCA variant CCcon.19 has acquired sCD4 sensitivity

Because passage of HIV-1 primary viruses in T-cell lines has long been known to cause the acquisition of sCD4 sensitivity (Daar et al., 1990; Gomatos et al., 1990), we determined whether the prolonged culture of CC1/85 in PBMC had had a similar effect on its phenotype. Clearly, CCcon.19 was much more sensitive than CC1/85 to sCD4; in a PBMC-based replication assay, an approximately 100-fold lower concentration of sCD4 was required to neutralize CCcon.19 than CC1/85 (Fig. 3A, Table 1). CC101.19, a CC1/85-derived virus that was passaged in PBMC from the same donors in the presence of increasing concentrations of AD101, remained as resistant as the input CC1/85 virus to sCD4 (data not shown).

To see whether the decreased resistance of CCcon.19 to sCD4 was specific for this agent, we also tested the broadly neutralizing anti-gp120 MAb b12 to an epitope overlapping the CD4BS. The b12 MAb neutralized CCcon.19 at an approximately 10-fold lower concentration compared to CC1/85 (Fig. 3B, Table 1). However, the neutralizing concentrations of the anti-gp120 MAbs 2G12 and 447-52D and the anti-gp41 MAbs 2F5 and 4E10 were similar for CC1/85 and CCcon.19 (Figs. 3C–E, Table 1). CCcon.19 had also not acquired sensitivity to MAbs 205-42-15, 205-43-1, and 205-46-9 that recognize conserved CD4BS-associated epitopes but which are able to neutralize only TCLA viruses and not primary isolates (Table 1) (Fouts et al., 1998). Moreover, CC1/85 and CCcon.19 were inhibited by similar concentrations of the gp41-peptide-based fusion inhibitor T-1249

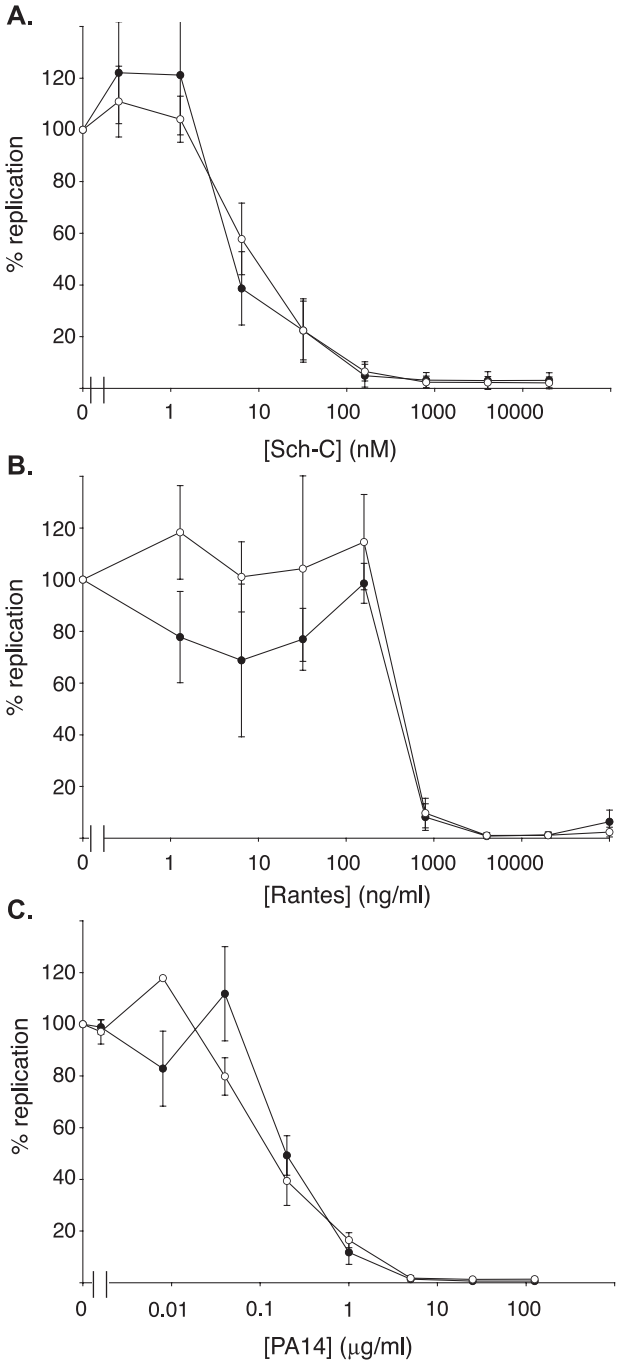

Fig. 2. The PTCA virus CCcon.19 is sensitive to CCR5 inhibitors. Isolates CC1/85 (filled circles) and CCcon.19 (open circles) were tested for their sensitivity in primary $CD4^+$ T cells to (A) SCH-C, (B) RANTES, and (C) MAb PA14. The extent of virus replication is presented as a percentage of p24 antigen production in the control culture (defined as 100%). The error bars represent the SEM of values from three independent experiments.

(Fig. 3F, Table 1). Thus, prolonged replication of the CC1/85 isolate in PBMC was not associated with the acquisition of a global sensitivity to neutralizing antibodies and entry inhibitors; the phenotypic changes were instead limited to the CD4BS itself and, to a lesser extent, to the topologically proximal epitope for the b12 MAb.

Changes in the env gene are responsible for sCD4 sensitivity

To assess what might be the genetic cause of the phenotypic change, we first sequenced the *env* genes from viruses isolated during the PBMC culture of CC1/85. For reasons of sample availability, we obtained genetic information from a virus sample, CCcon.20, which was frozen after 20 weeks, not 19. Analysis of the sequences from 12 individual clones showed that the only consistent changes during the evolution of CCcon.20 were in the V2 loop of gp120 (Fig. 4 and data not shown). Specifically, the originally dominant IRD sequence at positions 165–167 in CC1/85 (seven of eight clones) was substituted by KRN in all 12 CCcon.20 clones. These two changes increased the positive charge of the V2 loop by adding a lysine and removing an aspartic acid. The SN sequence at positions 188–189 of CC1/85 (seven of eight clones) was also deleted in all 12 CCcon.20 clones, disrupting the canonical motif NXT for glycosylation of residue N-189. Variants containing N-167 and lacking SN at positions 188 and 189 were both present in the input CC1/85 virus population at low frequency (one of eight clones in each case). Hence, these minor variants were presumably selected for during the prolonged PBMC culture (Fig. 4). However, none of the sequenced CC1/85 input viruses contained K at position 165, so the appearance of this residue in CCcon.20 is due either to a de novo mutation or the expansion of a variant that was initially present at an undetectably low frequency (<1 in 8 clones).

Table 1
Inhibition of HIV-1 isolates CC1/85 and CCcon.19 by a panel of inhibitors in primary $CD4^+$ T cells

Inhibitor (target)	IC_{50} for CC1/85 (μg/ml)[a]	IC_{50} for CCcon.19 (μg/ml)[a]
sCD4 (gp120 CD4bs)	>50	0.7
b12 (gp120 CD4bs)	6.0	0.3
2F5 (gp41)	>50	35.0
4E10 (gp41)	44.4	>50
2G12 (gp120)	1.8	4.2
447-52D (gp120 V3)	>50	>50
205-46-9 (gp120 CD4bs)	>50	>50
205-42-15 (gp120 CD4bs)	>50	>50
205-43-1 (gp120 CD4bs)	>50	>50
T1249 (gp41)	0.1	0.1
AD101 (CCR5)	<0.3[b]	<0.3[b]
SCH-C (CCR5)	3.7[b]	6.5[b]
AMD3100 (CXCR4)	>100[b]	>100[b]
PA14 (CCR5)	0.2	0.1
RANTES (CCR5)	0.05	0.04
2D7 (CCR5)	0.5	0.3
PA2 (CD4 D1)	0.6	>25
L222 (CD4 D1 CDR2)	0.4	2.8
Q4120 (CD4 D1 CDR1/2)	0.2	2.1
13B.8.2. (CD4 D1 CDR3)	0.2	5.0

[a] IC_{50} values for sCD4 inhibition curves were interpolated by fitting the data to a sigmoidal curve.
[b] In nanomolars.

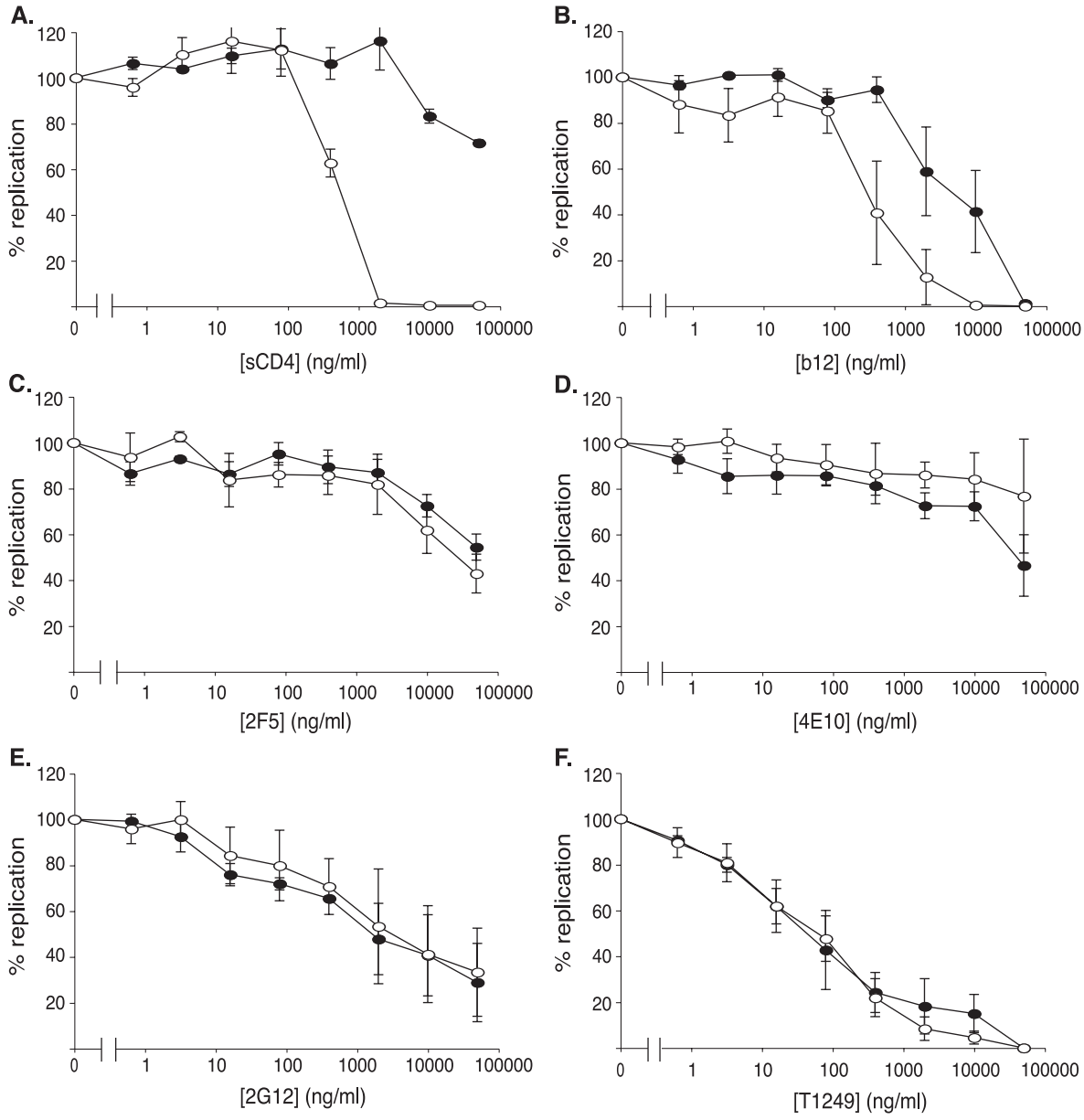

Fig. 3. The PTCA virus CCcon.19 has acquired sensitivity to sCD4 and MAb b12. Isolates CC1/85 (filled circles) and CCcon.19 (open circles) were tested for their sensitivity in primary $CD4^+$ T cells to (A) sCD4, (B) b12, (C) 2F5, (D) 4E10, (E) 2G12, and (F) T1249. The extent of virus replication is presented as a percentage of p24 antigen production in the control culture (defined as 100%). The error bars represent the SEM of values from three independent experiments.

We also examined the V2 sequences of clones derived from the CC1/85 culture at intermediate time points. By passage 10, all of the 11 sequenced CCcon.10 clones contained at least one of the I165K and D167N substitutions, and 2 of the clones carried both changes (Fig. 4). The glycosylation site at position 189 was also absent from 10 of the 11 CCcon.10 clones. Hence, 2 of the 11 CCcon.10 clones carried all three changes that were later found in all 12 of the CCcon.20 clones (Fig. 4). The double and triple mutants could have arisen either via recombination or through serial base substitution and deletion. None of the V2 changes were present in the AD101 escape mutant isolate CC101.19, consistent with its retention of sCD4 resistance (data not shown) (Kuhmann et al., in press; Trkola et al., 2002).

To assess whether the above changes in the V2 loop were sufficient to account for the phenotypic differences between CC1/85 and CCcon.19, we first created chimeric viruses based on HIV-1 NL4-3. These chimeras contained various *env* genes cloned from the isolates but were otherwise isogenic. The *env* gene of CC1/85 isolate displayed considerable sequence heterogeneity, so we made four infectious chimeras from this virus to try to cover the range of possible genotypes present. Because the sequence of the CCcon.19 isolate was much more homogenous, a single infectious chimera was deemed sufficient for an analysis of its prop-

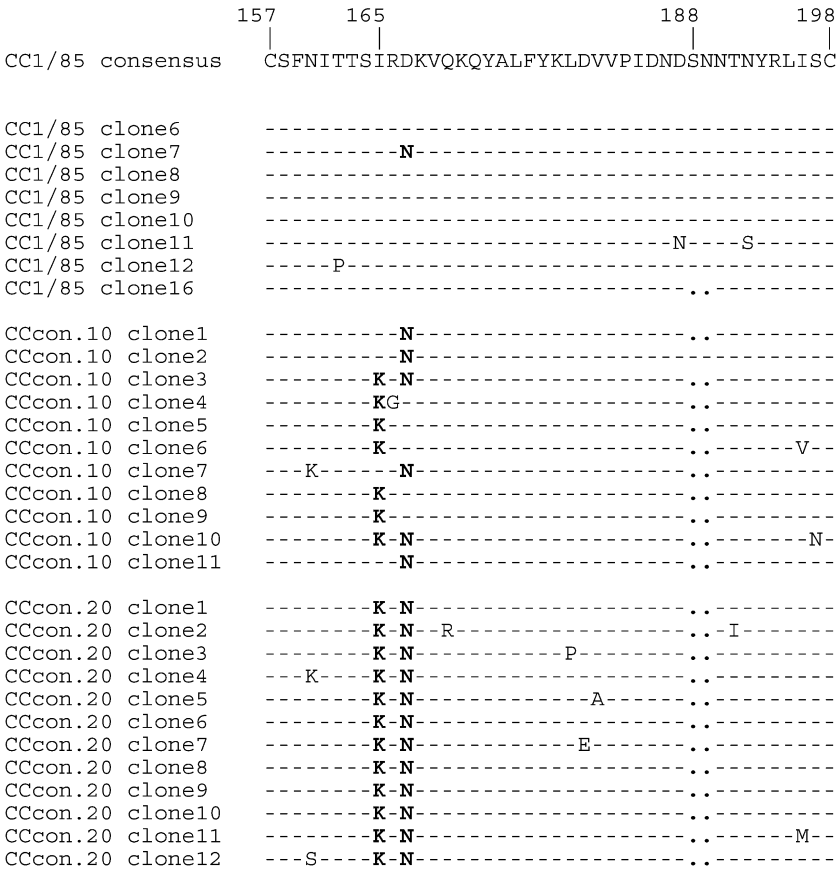

Fig. 4. Comparison of the gp120 V2 sequences from the CC1/85, CCcon.10, and CCcon.20 isolates. The top line shows the consensus V2 sequence of the CC1/85 isolate based on eight clones. The locations and numbers of specific amino acids, based on the HXBc2 sequence, are shown above the consensus line. For each clone, identity to the consensus sequence is designated by a dash, while periods indicate deletions and letters denote substitutions. Amino acid changes that appear to be selected for in the CCcon.10 and CCcon.20 viruses are highlighted in bold.

erties. The infectious chimera was actually made from PBMC frozen from the week-20 passage, and is designated CCcon.20 cl.11; we judged that the properties of CCcon.19 and CCcon.20 viruses would be very similar and most probably identical.

All five chimeric molecular clones were replication competent. Among the four CC1/85 clones tested, one of them, CC1/85 cl.7, was partially sensitive to sCD4 (IC_{50}, 1.1 μg/ml). The others, CC1/85 cl.6, CC1/85 cl.8, and CC1/85 cl.10, were fully resistant (Fig. 5A, Table 2). Of note is that CC1/85 cl.7 is the only one to contain residue N-167, as opposed to the more common D-167 (Fig. 4). The CCcon.20 cl.11 virus was sCD4-sensitive (IC_{50}, 0.04 μg/ml). Hence, not surprisingly, the determinants of sCD4 sensitivity lie within the *env* gene.

Site-directed mutagenesis of specific residues in the V2 loop

As noted above, the V2 loop of gp120 was the only region of Env where there were consistent differences between CC1/85 and CCcon.20 (Fig. 4). Furthermore, the partially sCD4-sensitive CC1/85 cl.7 virus was the only one of the four infectious CC1/85 clones to possess residue N-167 instead of the more common D-167 variant (Fig. 4). To assess whether this and other changes in the V2 region were responsible for sCD4 sensitivity, we made point substitutions in the *env* genes of the CC1/85 and CCcon.20 chimeric virus clones.

We first changed the IRD sequence at positions 165–167 in the sCD4-resistant CC1/85 cl.6 to the KRN motif found in the sCD4-sensitive CCcon.20 cl.11 virus to make the CC1/85 cl.6 KRN mutant. These changes caused a significant increase in sCD4 sensitivity, albeit not to the extent possessed by CCcon.20 cl.11 (Fig. 5B, Table 2). When back-substitutions were made in CCcon.20 cl.11, the single changes K165I (CCcon.20 cl.11 IRN) and N167D (CCcon.20 cl.11 KRD) were each sufficient to cause a 50-fold decrease in sCD4 sensitivity (Fig. 5C, Table 2). Moreover, the double mutant (CCcon.20 cl.11 IRD), containing both the K165I and the N167D changes, was 100-fold less sensitive to sCD4 (Fig. 5C, Table 2). Hence, this V2 loop motif has a substantial influence on sCD4 resistance.

We next introduced the N167D substitution into the partially sCD4-sensitive CC1/85 cl.7 virus to make CC/85 cl.7 IRD. This change was sufficient to convert the viral

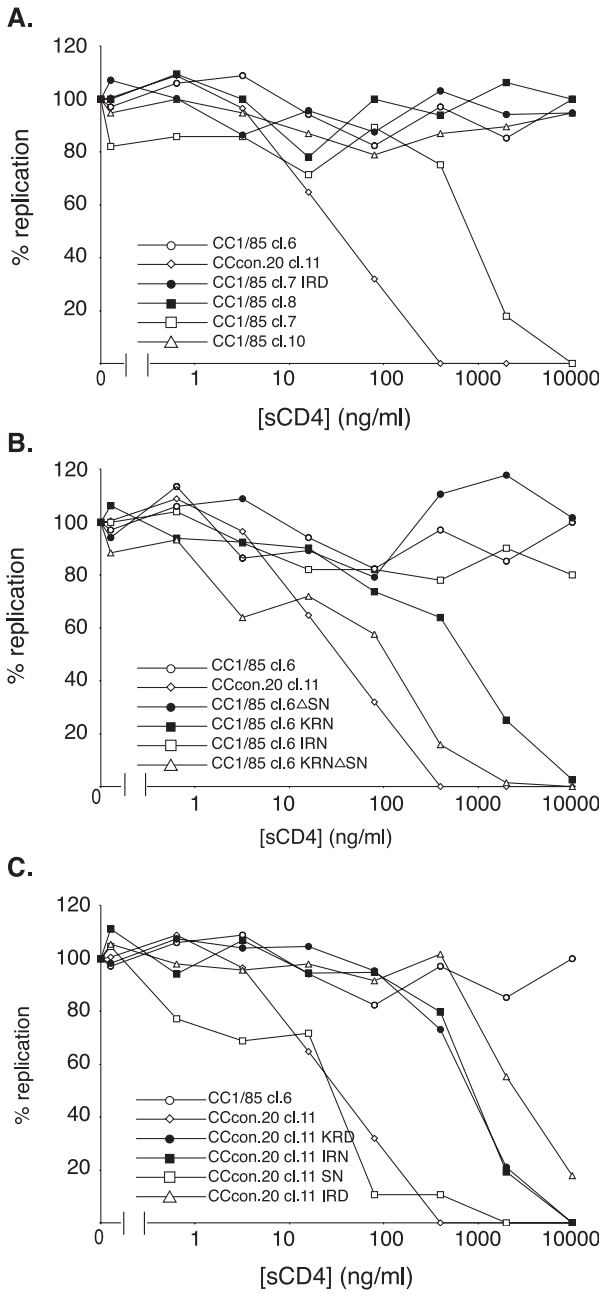

Fig. 5. Determinants of sCD4 sensitivity lie in the V2 region of HIV-1 gp120. Chimeric molecular clones were tested for sCD4 sensitivity in primary $CD4^+$ T cells. The extent of virus replication is presented as a percentage of p24 antigen production in the control cultures (defined as 100%). One representative experiment out of three is shown.

phenotype to complete sCD4 resistance (Fig. 5A, Table 2). However, making the converse D167N substitution in the sCD4-resistant CC1/85 cl.6 virus to create the CC1/85 cl.6 IRN clone did not create sCD4 sensitivity (Fig. 5B, Table 2). Thus, changes at residue 167 influence sCD4 sensitivity, but are not themselves sufficient to determine the overall phenotype.

In contrast, changes in CC1/85 cl.6 and CCcon.20 cl.11 that caused the deletion or insertion of the SN motif at positions 188–189, and the associated glycosylation site, had no direct influence on sCD4 sensitivity or resistance (Figs. 5B, C, Table 2). However, because the CC1/85 cl.6 KRN mutant was not fully sensitive to sCD4, we made the triple mutant CC1/85 cl.6 KRNΔSN to see whether the presence or absence of the glycosylation site at position N-189 indirectly influenced the phenotype. This triple mutant was as sensitive as CCcon.20 cl.11 to sCD4 (Fig. 5B, Table 2). Hence, the deletion of the SN sequence was necessary for complete sCD4 sensitivity, but only in the context of additional changes at residues 165–167.

CCcon.19 uses CD4 more efficiently

Because sCD4 was not present in the PBMC cultures, the acquisition of sCD4 sensitivity by CC1/85 must be secondary to a related change in viral phenotype that had other causes. It can be presumed that these reasons relate to increases in replication efficiency. One plausible explanation is that the interaction between virion-associated Env and cell surface CD4 became more efficient in culture, with a consequent but coincidental rise in the virion binding of sCD4. If so, then we reasoned that the sCD4-sensitive CCcon.19 virus should be less sensitive to inhibition by anti-CD4 MAbs; the MAbs should be less able to compete with the higher affinity interaction between CCcon.19 Env and cell surface CD4. To test this hypothesis, we determined the sensitivities of the CC1/85 and CCcon.19 isolates to anti-CD4 MAbs L222, 13B.8.2, Q4120, and PA2. Compared to CC1/85, the CCcon.19 virus was relatively resistant to all of these MAbs, by 7- to >40-fold (Figs. 6A, B, Table 1).

We also performed an ELISA using gp120 expressed from CCcon.20 and CC1/85 clones to assess whether the altered interaction between the CCcon.20 virus and CD4 was manifested at the level of monomeric gp120. In this assay, there was no difference in the binding of CD4-IgG2 to gp120 from CCcon.20 cl.11 compared to gp120s from

Table 2
Inhibition of HIV-1 clones by sCD4 in primary $CD4^+$ T cells

Virus	sCD4 IC_{50} (μg/ml)[a]
CC1/85 cl.6 (IRD)	>50
CC1/85 cl.7 (IRN)	1.1
CC1/85 cl.8 (IRD)	>50
CC1/85 cl.10 (IRD)	>50
CCcon.20 cl.11 (KRN)	0.04
CC1/85 cl.6 KRNΔSN	0.1
CC1/85 cl.6 KRN	1.0
CC1/85 cl.6 IRN	>50
CC1/85 cl.7 IRD	>50
CCcon.20 cl.11 KRD	1.1
CCcon.20 cl.11 IRN	1.2
CCcon.20 cl.11 IRD	3.1
CC1/85 cl.6 ΔSN	>50
CCcon.20 cl.11 SN	0.04

[a] IC_{50} values for sCD4 inhibition curves were interpolated by fitting the data to a sigmoidal curve.

CC1/85 cl.6 and CC1/85 cl. 7 (Fig. 6C). Thus, the half-maximal CD4-IgG2 binding concentrations for the three gp120s were 103, 100, and 57 ng/ml, respectively, which we consider to be comparable. The difference in the CD4 interaction of the CCcon.19 and CCcon.20 viruses compared to CC1/85 must therefore be due to changes in Env structure that operate at the level of native Env, and not monomeric gp120, as has generally been found in studies of sCD4 sensitivity changes (Ashkenazi et al., 1991; Brighty et al., 1991; Moore et al., 1992; Turner et al., 1992).

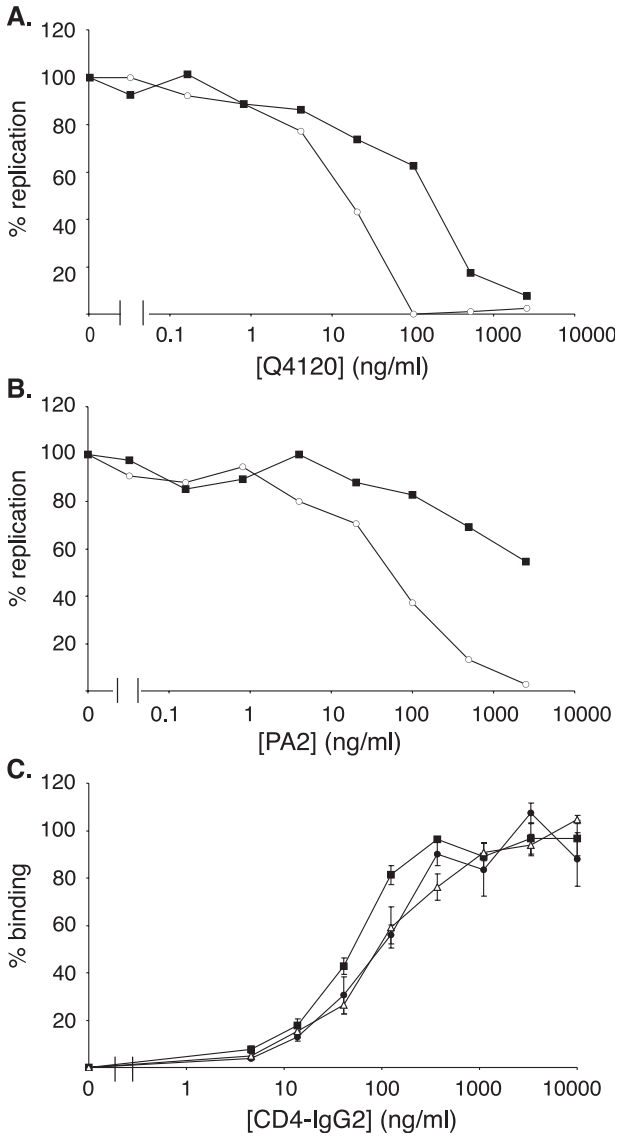

Fig. 6. CCcon.19 has evolved resistance to anti-CD4 MAbs without altering the affinity of monomeric gp120 for CD4. Isolates CC1/85 (open circles) and CCcon.19 (filled squares) were tested for their sensitivity in primary $CD4^+$ T cells to (A) MAb Q4120 and (B) MAb PA2. The extent of virus replication is presented as a percentage of p24 antigen production in the control culture (defined as 100%). (C) The binding of CD4-IgG2 to gp120 derived from clones CC1/85 cl.6 (closed circles), CCcon.20 cl.11 (open triangles), and CC1/85 cl.7 (closed squares) was measured by ELISA. The error bars represent the SEM of values from three independent experiments.

Table 3
Replication of CC1/85 and CCcon.19 in $CD4^+$ T cells and macrophages

log ($TCID_{50}$ macrophages)/log($TCID_{50}$ $CD4^+$ T cells)									
Isolate	Donor 1			Donor 2			Donor 3		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
CC1/85	0.00	0.00	0.67	0.00	0.23	0.63	0.67	0.67	0.96
CCcon.19	0.00	0.44	0.86	0.41	1.03	1.03	0.71	0.86	1.10

Values represent the ratio of infectious titers derived from macrophages and $CD4^+$ T cells for the indicated viruses on days 7, 14, and 21.

Replication of CCcon.19 in macrophages

To determine whether CCcon.19 had expanded its cell tropism compared to CC1/85, we measured the ability of both viruses to infect macrophages. There was a modest increase in the virus titer derived from macrophages infected with CCcon.19 compared to CC1/85 (Table 3). Thus, passage of the CC1/85 isolate in primary cells did increase its capacity for replication in macrophages. The levels of CD4 and CCR5 are significantly lower on macrophages compared to $CD4^+$ T cells (Di Marzio et al., 1998; Lee et al., 1999; Lewin et al., 1996; Tuttle et al., 1998). Hence, the above results are consistent with CCcon.19 having acquired a greater affinity for CD4 during passage. We could detect no difference in the entry efficiencies of the two isolates. However, we suspect that any increase in the efficiency of CCcon.19 entry is minor because it took over 10 weeks, or 35 replication cycles, in culture for the selective advantage of the preexisting variants in the viral population to become apparent (Fig. 4).

Discussion

Significant phenotypic changes occur when HIV-1 adapts to growth in T-cell lines, including the acquisition of abnormal sensitivity to NAbs (Moore and Ho, 1995). Neutralization resistance is required for viral persistence in the face of humoral immunity in vivo, but is lost upon in vitro adaptation. It has been argued that the various defense mechanisms used to ward off the binding of NAbs in vivo carry a price tag: a reduction in the rate of receptor-binding and fusion (Moore and Ho, 1995). Most NAbs impair Env–receptor interactions (Ugolini et al., 1997), so it seems plausible that mechanisms hindering antibody binding would also impede the attachment of similarly sized receptors to closely proximal gp120 sites. A different, but not mutually exclusive, possibility is that adaptation to T-cell lines reflects an adjustment to their reduced CD4 expression compared to primary $CD4^+$ T cells in vitro and in vivo (Platt et al., 2000). Thus, a selection pressure may favor Env variants with a higher CD4 affinity when HIV-1 is cultured

on T-cell lines, and the altered Env structures may, coincidentally, have more exposed neutralization epitopes, particularly those associated with the CD4BS (Platt et al., 2000). Under both scenarios, the characteristic increase in the sensitivity of TCLA viruses to sCD4 is coincidental to Env alterations that open up the CD4BS but that would be disfavored in the face of an effective humoral response in vivo. Resistance to sCD4 is itself a consequence of the natural mechanisms used in vivo to thwart the actions of NAbs to the CD4BS (Moore and Sweet, 1993; Moore et al., 1993).

Although the above two scenarios are related, they can be distinguished by their differing requirements for a reduced CD4 density on target cells in vitro. If the primary driving force for change is to reduce Env's armor against NAbs, it should not matter whether the CD4 density differs in vitro and in vivo. However, if the dominant selection pressure is for use of the lower CD4 levels on T-cell lines, neutralization sensitivity would not arise in vitro when primary $CD4^+$ T cells are the targets (Platt et al., 2000). Our demonstration that a primary HIV 1 isolate can, in fact, acquire sCD4 sensitivity when cultured only on PBMC therefore shows that a lower CD4 density is not obligatory for reduced neutralization resistance.

The overall selection pressure on HIV 1 during in vitro adaptation might still involve increasing its CD4 binding efficiency, by altering the CD4BS configuration to increase its accessibility to CD4 and, by coincidence, sCD4. The acquired resistance of CCcon.19 to anti-CD4 D1 MAbs is consistent with this hypothesis. Conversely, selection for sCD4 and CD4-Ig resistance created increased sensitivity to anti-CD4 D1 MAbs (Klasse and McKeating, 1993). The modestly increased ability of CCcon.19 to replicate in macrophages is also relevant; macrophages express less CD4 than $CD4^+$ T cells (Lee et al., 1999; Lewin et al., 1996).

The neutralization-sensitive TCLA virus HIV-1 IIIB became neutralization resistant after adaptation to growth in an accidentally infected human (Beaumont et al., 2001). The dominant selection pressure in vivo must have been for NAb resistance. However, when NAbs are absent, either in vitro or when the immune system is substantially impaired, the balance shifts away from the need for protection. HIV-1 and SIV variants with increased replication competence evolve in the later stages of infection in vivo (Kimata et al., 1999; Kwa et al., 2003). Perhaps these phenotypic changes reflect, at least in part, a relaxation in Env's defenses as the NAb response diminishes during disease progression? In vitro, adaptations creating neutralization sensitivity are not restricted to HIV-1, but occur also with SIV, FIV, and EIAV (Baldinotti et al., 1994; Cook et al., 1995; Means et al., 1997; Montefiori et al., 1998b). The general principle of a balance between binding cellular receptors and NAbs would be fundamental to all persistent lentiviruses, irrespective of their specific receptor(s). Hence, different viruses might respond similarly to replication in cell lines that differ significantly from the natural target cells in respect of the concentrations and identities of cell surface receptors and attachment factors (Ibrahim et al., 1999; Mondor et al., 1998; Patel et al., 1993; Spenlehauer et al., 2001). The efficiency of envelope glycoproteins incorporation into the virion may be another factor that could vary with the producer cell, and this too could affect the neutralization sensitivity of any lentivirus (Klasse and Moore, 1996).

Previous studies on HIV-1 adaptation in vitro have been complicated by co-receptor switching or selection during the process. Most T-cell lines express CXCR4 but lack CCR5, whereas CCR5 is more commonly used in vivo (Clapham and McKnight, 2002; Doms, 2000; Moore et al., in press; Pohlmann and Doms, 2002). Isolation of HIV-1 on activated PBMC usually favors R5 virus replication; on T-cell lines, X4 viruses (Moore et al., in press). Hence, TCLA viruses are not just neutralization-sensitive, they differ in co-receptor usage from most primary isolates. Most, but not all, studies indicate that co-receptor usage is not a major influence on neutralization sensitivity; primary X4 and R5 isolates all tend to be neutralization-resistant (LaCasse et al., 1998; Montefiori et al., 1998a; Polonis et al., 2003; Trkola et al., 1998a). However, it remained possible that an adapted virus would acquire neutralization sensitivity in vitro only if it used CXCR4. Clearly, we now show this is not the case. The sCD4-sensitive, PBMC-adapted virus CCcon.19 retained its original R5 phenotype, and does not use CXCR4. The acquisition of sCD4 sensitivity is not, therefore, just a consequence of T-cell line adaptation, it can occur after prolonged in vitro passage in primary cells and without co-receptor switching. The R5 PTCA variant has at least one property, sCD4 sensitivity, resembling those of classical X4 TCLA viruses. Hence, HIV-1 "laboratory adaptation" can involve more than just a switch in co-receptor usage or selection of variants that use only CXCR4. Whether this will be true of all PTCA viruses remains to be determined.

Most TCLA viruses have acquired an epitope-independent, global sensitivity to NAbs. This applies even to anti-gp41 NAbs that act after receptor binding (Moulard et al., 2002). It is still not fully understood why. Studies on one experimental system suggest that changes in the gp41–gp120 interaction can be associated with an increased exposure of multiple antibody epitopes (Park and Quinnan, 1999; Park et al., 2000). However, changes in only the V2 loop were observed in the PTCA virus CCcon.19. It became more sensitive to sCD4 and, to a lesser extent, the CD4BS MAb, b12, but not to NAbs 2G12, 2F5, and 4E10. Hence, the underlying mechanism is specific to the CD4BS, but even here its effect is limited as CCcon.19 did not become sensitive to CD4BS MAbs 205-42-15, 205-43-1, and 205-46-9. Acquisition of global neutralization sensitivity is not, therefore, an obligatory consequence of in vitro adaptation.

The entry inhibitors AD101, SCH-C, and T-1249 had similar potencies against the PTCA virus CCcon.19 and the

parental CC1/85 virus. Although we did not measure fusion rates directly, other studies imply that the overall fusion kinetics of CCcon.19 will probably not be significantly altered by its acquisition of sCD4 sensitivity (Reeves et al., 2002). The unaltered sensitivity to AD101, SCH-C, and T-1249 is consistent with their known actions at stages after CD4 binding (Melikyan et al., 2000; Moore and Doms, 2003).

Genetic sequencing revealed that the only consistent differences between CC1/85 and CCcon.20 were in V2, specifically at residues 165–167 and 188–189. The latter change eliminates a canonical N-linked glycan site. The gradual evolution of these changes is apparent by inspection of sequences of viruses from passage 10 (Fig. 4). Between CC1/85 and CCcon.20, there were no relevant sequence alterations in the gp120 core. This is not surprising because the core sequences of primary and TCLA viruses are very similar (Kwong et al., 2000). However, the variable loops in general and the V2 domain in particular are major determinants of the TCLA phenotype. For example, grafting the V1/V2 loop structure from a primary virus onto a TCLA strain creates a chimeric virus with properties resembling those of the primary virus (Koito et al., 1994; Morikita et al., 1997). One role of the V2 loop is to shield the underlying, more conserved receptor binding sites from NAbs; the V2 loop is dispensable for HIV-1 replication in vitro, at least in some cell types, but V2 loop-deleted viruses are very neutralization-sensitive (Cao et al., 1997; Stamatatos et al., 1998; Wyatt et al., 1995). The V2 loop conformation may influence CD4 binding because of its predicted spatial proximity to the recessed cavity that is a critical CD4BS element (Wyatt et al., 1998). Several V2 residues influence the binding of MAb b12, at least in the context of other primary R5 viruses (Mo et al., 1997; Pantophlet et al., 2003).

Substitutions at residues 165–167 during adaptation of various HIV-1 strains to replication in vitro have been reported previously (Table 4) (Beaumont et al., 2001; Bouma et al., 2003; Follis et al., 1998; Hofmann-Lehmann et al., 2002; Mo et al., 1997; Wrin et al., 1995). Overall, adaptation is associated with an increase in the positive charge of this amino acid motif: IRD to IRN (Mo et al., 1997), IRD to IGN (Bouma et al., 2003), IRD to RRD (Wrin et al., 1995), IRD to KRD (Follis et al., 1998). In our study, the change was IRD to KRN, again increasing the positive charge of the motif. Hence, different HIV-1 strains with various phenotypes may take a similar genetic pathway when adapting to growth in vitro. The D167N substitution also arose in the JR-CSF strain when this virus became resistant to MAb b12 in vitro (Mo et al., 1997). By itself, that substitution was insufficient to confer b12 resistance, just as it was not enough to confer b12 sensitivity in the present study. Residue 167 may be involved in interactions with other amino acids more proximal to the CD4BS and its associated epitopes. Such an indirect influence would be consistent with previous findings on the determinants of neutralization sensitivity and resistance (Si et al., 2001; Ye et al., 2000).

Table 4
Variation in residues 165–167 of HIV-1 gp120 during adaptation to replication in vitro

Parental strain[a]	Tropism[b]	Residues 165–167 (Pre)[c]	Adaptation system[d]	Residues 165–167 (post)[e]	Reference[f]
Case C1/85	R5	IRD	PBMC	KRN	present study
JRCSF	R5	IRD	PBMC	IRN	Mo et al., 1997
MN	X4	IRD	H9	IGN	Bouma et al., 2003
168P	X4	IRD	H9	RRD	Wrin et al., 1995
320SI	X4	IRD	MT4/H9	KRD	Follis et al., 1998
IIIB	X4	IRG	in vivo (human)	IKG	Beaumont et al., 2001
SHIV vpu+	X4	IRG	in vivo (macaque)	IGE	Hofmann-Lehmann et al., 2002

[a] Virus designation.
[b] Tropism (R5 or X4).
[c] Identity of residues 165–167 (HxB2 numbering system) before adaptation.
[d] Experimental system used for adaptation.
[e] Identity of residues 165–167 after adaptation.
[f] Source of information.

The gp120 protein is heavily glycosylated, which is one of HIV-1's NAb evasion and escape mechanisms (Johnson and Desrosiers, 2002; Olofsson and Hansen, 1998; Reitter et al., 1998; Wei et al., 2003). The loss of glycans can result in faster replication kinetics, at least in vitro, particularly when sites near the CD4BS are disrupted (Ohgimoto et al., 1998). The glycan lost in CCcon.20 is thought likely to physically abut the CD4BS (Wyatt et al., 1998). A plausible explanation of the sequence changes in CC1/85 that create CCcon.20 is, therefore, that they alter the geometry of the V1/V2 loop structure in relation to the underlying CD4BS. Both the loss of the glycan at position 189 and a change in the positioning of the V1/V2 loop as a whole may be relevant. The structural consequences are likely to be manifested only at the level of the functional Env complex, and inapparent from studies with monomeric gp120 from HIV-1 (Ashkenazi et al., 1991; Brighty et al., 1991; Moore et al., 1992; Turner et al., 1992) and HIV-2 (Mulligan et al., 1992).

The principal driving force behind adaptation would be the facilitation of cell surface CD4 binding, the increase in sCD4 and b12 sensitivity arising from the increased accessibility of the CD4BS and its associated epitopes to ligands. In vivo, this effect would be countered by NAbs to CD4BS epitopes. The ability of HIV-1 to replicate in cell types such as the macrophage might also be relevant in vivo, particularly when humoral immunity becomes impaired in the later stages of infection (Igarashi et al., 2001). Macrophages express less CD4 than T cells (Lee et al., 1999; Lewin et al., 1996), so it is conceivable the Env complex might not always be configured optimally for entry into both cell types simultaneously. Any minor increments in replication effi-

ciency in macrophages might have a significant effect when amplified over multiple cycles (Coffin, 1986). Overall, the efficiency of HIV-1 entry in vivo is subject to the outcome of the exquisite interplay of opposing influences on the highly plastic configuration of the Env complex.

Methods

Antibodies and other reagents

The CC-chemokine RANTES was obtained from PeproTech Inc. (Rocky Hill, NJ). The anti-CD4 MAb PA2, the anti-gp120 MAb PA14, the sCD4 and CD4-IgG2 proteins, and the gp41-based peptide T1249 were all gifts from Dr. William Olson (Progenics, Tarrytown, NY) (Allaway et al., 1995; Kilby and Eron, 2003; Olson et al., 1999). The anti-Env MAbs 2F5, 4E10, and 2G12 were donated by Dr. Hermann Katinger (Polymun Scientific Inc, Vienna, Austria) (Trkola et al., 1995), the anti-gp120 MAb b12 by Dr. Dennis Burton (Scripps Research Institute, La Jolla, CA) (Burton et al., 1994), and the anti-gp120 MAbs 205-42-15, 205-43-1, and 205-46-9 by Dr. Michael Fung (Tanox Inc, Houston, TX) (Fouts et al., 1998). The anti-gp120 MAb 447-52D was obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH, contributed by S. Zolla-Pazner. The small molecule CCR5 and CXCR4 inhibitors AD101, SCH-C, and AMD3100 were provided by Dr. Bahige Baroudy (Schering Plough Research Institute, Kenilworth, NJ) (Donzella et al., 1998; Strizki et al., 2001; Trkola et al., 2002). Anti-CD4 MAbs L222, Q4120, and 13B.8.2 were gifts from Dr. Quentin Sattentau (Oxford University, UK) (Davis et al., 1992; Healey et al., 1990; Sattentau et al., 1989).

Generation of the HIV-1 CC1/85 variant, CCcon.19

The R5 primary HIV-1 isolate CC1/85 was derived in January 1985 from individual "Case C" who was enrolled in a New York Blood Center cohort and shown retrospectively to have become HIV-1 seropositive between October 1979 and February 1980 (Connor et al., 1993, 1997). The conditions used to generate the CCcon.19 variant have been described in detail elsewhere (Trkola et al., 2002). Briefly, CC1/85 was added at one thousand 50% tissue culture infectious doses ($TCID_{50}$) per ml to 20 ml of mitogen-activated PBMC (1×10^6/ml). The culture was passaged weekly, using PBMC from a different, anonymous donor each week. The production of p24 antigen was monitored regularly to ensure that HIV-1 replication was occurring. At least once every 2 weeks, the culture supernatant was frozen for later sensitivity and co-receptor utilization studies.

Infection of $CD4^+$ T cells and macrophages

Mitogen-activated, $CD8^+$ T-cell-depleted PBMC (referred to hereafter as primary $CD4^+$ T cells) were prepared from leukopacks pooled from the blood of four healthy volunteers. The pooled fractions were treated with the "RosetteSep $CD8^+$ depletion cocktail" (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions, before Ficoll density gradient separation of leukocytes to deplete $CD8^+$ T cells from the PBMC by rosette formation (Trkola et al., 1998b). The $CD8^+$ T-cell-depleted leukocytes were then stimulated as described elsewhere (Trkola et al., 1998b) and used for HIV-1 replication assays 3 days later (Trkola et al., 2002).

Macrophages were isolated, then infected with HIV-1 as described previously (Ketas et al., 2003). Culture supernatants were harvested at days 7, 14, and 21 for measurement of p24 antigen concentrations.

Cloning of env genes

env clones were named according to our previous nomenclature (Trkola et al., 2002). Thus, CC1/85 is the parental isolate, and CCcon.19 refers to the control virus that was cultured for 19 passages in PBMC. The clones generated and used here extend this nomenclature, such that CC1/85 cl.XX refers to clone XX from the parental CC1/85 isolate. The nomenclature used to designate the other clones generated in this study was also derived from that used elsewhere (Kuhmann et al., in press; Trkola et al., 2002).

env genes were cloned into the vector pAMP1 using the CloneAmp system for rapid cloning of amplification products (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The PCR products to be cloned were generated by nested PCR from genomic DNA that was isolated from infected PBMC by methods used previously (Kuhmann et al., in press; Trkola et al., 2002). The GenBank accession numbers for the sequences are AY357338 through AY357345 (CC1/85), AY357464 through AY357469 (CC101.19), AY357525 through AY357535 (CCcon.10) and AY357536 through AY357547 (CCcon.20).

Recombinant gp120 proteins from clones CC1/85 cl.6, CC1/85 cl.7, and CCcon.20 cl.11 were produced as described previously (Kuhmann et al., in press).

Construction of chimeric NL4-3/env proviruses and site-directed mutagenesis

Chimeric proviruses were constructed from the pNL4-3 proviral plasmid (Adachi et al., 1986) (AIDS Research and Reference Reagent Program, NIAID, NIH, contributed by Dr. M. Martin) by overlapping PCR, described previously (Kuhmann et al., in press).

Site-directed mutagenesis of the *env* clones was performed with the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions, using the pBluescript(KS+) plasmids that contained the *Eco*RI to *Xho*I fragments. All of these *env* constructs were sequenced to confirm that the desired changes were present without other mutations, then the *Eco*RI to *Xho*I

fragments were subcloned into pNL4-3 as described above.

Infectivity of chimeric viruses

Clonal, replication-competent, chimeric NL4-3/*env* viruses were prepared by transfection of 293T cells, which were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (complete medium). Briefly, 15 μg of the full-length proviral plasmid was transfected into 1×10^7 293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The cells were washed after 24 h, then fed with complete medium. The supernatants were collected 48 h post-transfection, filtered through a 0.45-μm filter, and stored in aliquots at −80 °C. These virus stocks were titered on primary $CD4^+$ T cells, then replication assays were performed as described for standard HIV-1 isolates in PBMC (Trkola et al., 2002), except that 3000 $TCID_{50}$ per ml of virus were used for infection. The culture supernatants were analyzed for p24 production after 10 days. The infectivities of viruses produced by transient transfection were determined on day 12 postinfection in primary $CD4^+$ T cells, and ranged from 1×10^4 to 1×10^5 $TCID_{50}$/ml of viral supernatant. In addition, the p24 production in the primary $CD4^+$ T-cell replication assays was similar for all the clonal viruses used in this study, typically 5–10 ng/ml of p24.

Binding of gp120 to CD4-IgG2

The gp120 capture enzyme-linked immunsorbent assays (ELISA) were performed essentially as described elsewhere (Moore and Sodroski, 1996). The gp120 proteins were captured at saturating concentrations to plastic plates coated with the sheep polyclonal antibody D7324 to the C-terminus of gp120, then the binding of CD4-IgG2 was detected (Moore and Sodroski, 1996). The background OD_{490} signal (no gp120) was subtracted from the signal derived using gp120 at each input MAb or CD4-IgG2 concentration. The net OD_{492} value obtained at 10 μg/ml of CD4-IgG2 was defined as 100% for normalization purposes.

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